JCB

Mechanosensitive calcium flashes promote sustained RhoA activation during tight junction remodeling

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June 17, 2021

Re: JCB manuscript #202105107

Dr. Ann L Miller
University of Michigan-Ann Arbor
Molecular, Cellular, and Developmental Biology
5264 Biological Sciences Building
1105 North University Ave.
Ann Arbor, MI 48109-1085

Dear Dr. Miller,

Thank you for submitting your manuscript entitled "Mechanosensitive calcium signaling promotes epithelial tight junction remodeling by activating RhoA" to Journal of Cell Biology. The manuscript has now been assessed by expert reviewers, whose reports are appended below. Unfortunately, after an assessment of the reviewer feedback, our editorial decision is against publication in JCB.

As you can see, the reviewers' enthusiasm was mixed, but the consensus was that considerably more mechanistic insight and characterization would be needed into the direct causality of the links between membrane tension, tight junction integrity, calcium flashes, and the activation of Rho flares. In fact, at the original editorial review stage (prior to sending out for peer reviewing), concerns were expressed that this study was borderline and would require additional data that would have to expand it, moving it out of the Report category.

Our impression from the peer reviewing by three experts known for expertise in differing facets of your study is that - unless you already have considerable additional mechanistic information - it would not be practical for this paper to be revised within a reasonable JCB resubmission period. Although we would support your not needing to explore research directions not clearly relevant to the key conceptual advance (such as needing to divert to explore PKC), it seems clear from the reviewing that much more experimental work would be needed. It might also help to note that even though the calcium-Rho flare link seems intriguing, Reports need to be definitive and firmly establish an exciting new direction, rather than just being a short Article.

Of course, if you do have data which can address all of these remaining concerns in full, you are welcome to appeal this decision. However, given the scope and breadth of the necessary revisions, we would recommend providing a revision plan prior to undertaking any revisions.

We regret that the intriguing initial findings you report apparently linking calcium and small GTPase flare signaling did not currently meet the high requirements of the JCB Report and hope that you can in-depth characterize this intriguing concept in the future.

Please also note that our journal office will transfer your reviewer comments to another journal upon request.

Thank you for your interest in Journal of Cell Biology.

With kind regards,

Kenneth Yamada, MD, PhD
Senior Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

The Miller lab recently (2019) reported that flashes of Rho signaling at cell-cell junctions are responsible for repairing defects in tight junction (TJ) integrity. The present manuscript develops from this finding, to probe the upstream mechanisms that are responsible for activating Rho when TJ defects appear. Prompted by evidence that intracellular calcium signaling can support Rho in other contexts, the authors identify a role for dynamic calcium transients at sites of TJ disruption and repair and implicate

mechanosensitive cation channels in this calcium flux, by applying the spider venom toxin GsMTx4. They propose a pathway where mechanosensitive flux of calcium activates Rho to drive TJ repair.

This is an interesting report that is based on data of superb quality. However, I am not sure that it is quite at the level of a JCB Report, at least in its present form.

I have two major issues (which are the important ones for me, although there are some specific questions/issues below).

1) Establishing the causal relationship between calcium and Rho. The authors reasonably show a role for calcium in Rho signaling, but I am not sure that their linear model (Fig 5H) is fully supported.
I am puzzled that GsMTx4 increased the frequency of Rho flares. In the linear pathway that the authors present (Fig 5H), if calcium signaling were necessary for RhoA to be activated, I would have predicted a decrease in frequency of Rho flares (as well as the decrease in amplitude and duration that the authors document). This disjunction would suggest, to me, that calcium may not be upstream of Rho activation, but rather act at some stage to sustain or amplify the Rho signal, once it is initiated. (Along these lines, what does the combination of BAPTA-AM and 2-APB do to the frequency of Rho flares?)

2) New knowledge. I think that it is important to understand how the Rho flares are evoked, but the present analysis applies currently-available concepts (from wound healing, cell migration etc) to the specific instance of TJ repair. Given the prevalence of this model, it is perhaps not surprising that it works in the present instance.
For a JCB Report, I would ask for some new conceptual insights. One line to pursue could be the issue of calcium and RhoA activation that I raised above. Another would be to probe the notion that changes in membrane tension are key at sites of TJ dysfunction. The authors base this hypothesis on observed deformations of the apical membrane at sites of dysfunction and the loss of a potential actin-membrane coupler in ZO proteins. But this is not addressed in greater depth. (One could imagine, for example, that loss of membrane-actin coupling would decrease the tension that is applied to the bilayer from the cortex - and the protrusions reflect a relaxed ballooning of the membrane.) FRET sensors for membrane/bilayer are now available and could be tried (though I appreciate that their application is not a trivial matter).

Specific (more technical) points

a) Fig 2E. Can the authors analyse the relationship between calcium flash and Rho response statistically? At least by eye, I do see some high-ish calcium responses that don't give very impressive RhoA responses and lowish calcium responses that seem to link to large RhoA responses.

b) Why did the authors inject GsMTx4? Can it be added to dechorionated embryos to shorten the incubation time? (To reduce secondary effects that might have arisen from relatively prolonged MSC inhibition.)

Reviewer #2 (Comments to the Authors (Required)):

Varadarajan et al study the role of intracellular calcium on epithelial barrier maintenance during development. This work is a continuation of previous findings from the Miller lab on epithelial barrier repair in actively remodelled epithelia (Stephenson et al 2019). Using Xenopus embryos, high resolution live imaging and pharmacological treatments, the authors show that intracellular calcium increase follows paracellular leaks. Furthermore, the authors show that intracellular calcium increase is dependent on the activity of mechanosensitive calcium channels and is necessary for Rho-flare mediated TJ repair. Lastly, the authors show that the mechanosensitive channel mediated calcium influx is necessary for epithelial barrier maintenance during normal development.
The strength of this work comes from the employment of high-resolution live imaging which led to the identification of a novel function of intracellular calcium. The identification of a novel role for intracellular calcium in epithelial barrier maintenance should be of interest to researchers across a broad range of disciplines.
Despite the novelty of this work, there are several issues that need to be addressed.

Major points:

1) One of the claims of this work is that intracellular calcium increase precedes RhoA activation during tight junction remodelling and is required for RhoA activation. However, the data presented in the manuscript are not consistent with this notion. Specifically, while the authors argue that calcium flashes precede RhoA activation at sites of tight junction breakage, in Figure 3B, 4C and Movie 2(600sec) there is a clear enrichment of active RhoA before the appearance of calcium elevation. I do not know if this has to do with the sensitivity of detection or kinetics of the probe however even when using the faster calcium reporter (Fig 2D and F) there is an initial increase in RhoA which is synchronous with the calcium elevation. Overall, I am not convinced that calcium elevation does in fact precede RhoA activation and it's just as likely that the opposite is true. Do we know how fast is the RhoA reporter's response and how it compares to the fast calcium reporter? Additionally, in Figures 3C, 4D and 5E there is enrichment of active RhoA at the site of tight junction breakage (albeit lower in intensity) in the absence of calcium

elevation. This shows that the RhoA activation at these sites isn't calcium-dependent but may be enhanced by calcium elevation. The authors should examine the possibility that initial RhoA activation precedes calcium influx and that subsequent calcium influx, possibly triggered by RhoA, further stimulates RhoA. Is RhoA activation necessary for calcium flashes during tight junction remodelling? The authors could use an optogenetic construct to activate RhoA specifically at the tight junctions to see if that elicits calcium and use RhoA/Rock inhibitors to see if they affect calcium flashes. Finally, can RhoA activation rescue the defective tight junction repair in embryos treated with GMSTX4 of 2AP+BAPTA? Again, an optogenetic construct to activate RhoA specifically at the tight junctions could be used here.

2) The authors show a clear correlation between tight junction leakages and calcium flashes. However, they don't directly assess the impact of tight junction leakage on intracellular calcium. The authors could examine the frequency of calcium flashes in control embryos and embryos treated with Latrunculin A which leads to increase of tight junction leakage events (Stephenson 2019). Ideally to directly establish a cause and effect relationship, calcium levels can be examined after TJ laser ablation.

3) The calcium regulated protein PKC has been shown to regulate tight junction assembly and the authors show that the C2 domain of PKC localises at the sites of tight junction leakages. Therefore, PKC might be a downstream effector of calcium during epithelial barrier maintenance. It would be interesting to pharmacologically inhibit PKC to examine the role of PKC in the context of breakage repair.

4) The data presented by the authors show that mechanosensitive calcium channels are responsible for localised calcium influx during paracellular leakages (localised C2-GFP recruitment) and are necessary for tight junction repair and epithelial barrier maintenance. Calcium flashes appear throughout the cell when imaged with the GCAMP6 calcium sensor. This uniform elevation of calcium within cells suggests that IP3 mediated calcium influx from the ER contributes to calcium flashes. This is supported by the fact that 2APB an IP3R inhibitor prevents calcium flashes during tight junction leakage. The authors should discuss or if possible test the possibility that an initial calcium entry through MSCs activates PLC near the plasma membrane, leading to the production of IP3s and subsequent activation of IP3R. Pharmacological PLC inhibition followed by examination of calcium flashes during tight junction remodelling will be beneficial. This will be a great addition to the manuscript since it will reveal if there is a need for production of IP3 downstream of MSCs for the generation of calcium flashes.

5) In Figure 2E the authors present a correlation between the levels of calcium and RhoA activation. Plotting calcium levels against active RhoA levels and analysis with a Pearson's correlation coefficient test is the proper way to quantitatively assess if there is a correlation between calcium levels and RhoA activity.

6) The authors argue that GMSTX4 treatment does not affect cell junctions. However, in contrast to the quantification in Fig. S3D', in Figure S3D PMLC appears dramatically reduced in GMSTX4 treated embryos. The reduction of PMLC in GMSTX4 treated embryos shown in Figure S3D argues that cell-cell junctions are affected by GSMTX4 treatment. The authors should stain control and GMSTX4 treated embryos for cell-cell junction markers C-cadherin, b-catenin and or a-catenin to make sure that GSMTX4 treatment does not affect cell-cell junctions.

7) The authors claim that defective epithelial barrier function (Figure 5A) results from the accumulation of defective repair of local leaks in GMSTX4 treated embryos. Another possibility may be that in GMSTX4 treated embryos the frequency of tight junction leaks increases, especially if cell-cell junctions are affected as shown in PMLC levels in Fig. S3D. To explore this possibility the authors could quantify the frequency of leakage events in control and GMSTX treated embryos. If the frequency of leakage events is unaffected then global epithelial barrier defects stem from the accumulation of defective repair of local leaks. If the frequency of leakage events is increase in GMSTX4 treated embryos, then the global tissue barrier defect stems from both increase of leakage events and accumulation of defective local repairs.

8) In Figure 3a the authors use 2APB+BAPTA AM to block calcium flashes. What happens to calcium flashes when the embryos are treated with only 2APB?

Specific concerns:

1) Maybe the authors can swap Fig1 C and D to better follow the flow of the text?

2) In Figures 1B and 2d the authors use a different approach to quantify Calcium (C2) and calcium (GCAMP6). Is there a reason for this? why is calcium signal quantification different?

3) In some examples like Fig2a, 2c-d only one cell next to the junction displays a calcium flash. Can the authors discuss this? Is calcium only required in one of the two cells repairing the TJ? Is there a difference in repair kinetics if only one of the two cells

displays calcium elevation? Could the authors introduce the RhoA sensor in a mosaic pattern and examine RhoA activation at breakpoints in cells that do display calcium vs ones that do not?

4) In figure 4a the authors show that membrane blebs correlate with RhoA flares, suggesting increase in local membrane tension. Is there any direct evidence that membrane blebs are sites of increased tension. Citing previous work like this one https://www.pnas.org/content/106/44/18581 will strengthen the authors' hypothesis.

5) In some of the time-lapse movies there are calcium flashes in cells away from tight junction leaks. Can the authors comment on this?

6) The authors are compressing the embryos during imaging. Are the breaks in part induced by this slight compression?

Reviewer #3 (Comments to the Authors (Required)):

Cell-cell junctions respond to different stimuli including mechanical ones that are known to regulate tight junction assembly/integrity via different types of signalling pathways including calcium increases and RhoA-regulated actomyosin remodelling. Previously, the Miller group has found that local leaks in the barrier are rapidly repaired by localized transient activation of RhoA that they call Rho flares.

This paper asks how Rho flares are initiated. They discover that intracellular calcium flashes occur in Xenopus laevis epithelial cells undergoing Rho flare-mediated remodelling of ZO-1. Depletion of intracellular calcium or inhibition of mechanosensitive calcium channels (MSC) reduced the amplitude of calcium flashes and diminished the activation of Rho flares. They propose a model in which MSC-dependent calcium flashes are important for epithelial cells to sense and respond to local barrier leaks induced by mechanical stimuli.
This paper is using a similar methodology as described by the same group in two previous papers to study Rho flares. The present paper remains at a descriptive level and solely relies on drugs/inhibitors to analyse the molecular mechanism of Rho activation. No attempts are made to identify the actual channel relevant for the calcium influx nor the molecular mechanism leading from calcium increases to RhoA activation. Given what is known about the roles of calcium and RhoA in tight junction formation, the current manuscript does not represent a significant advance. For example, ethanol-induced [Ca(2+)]i release, mediated by stimulating IP3R-gated Ca(2+) channel, activates Rho/ROCK in Caco-2 cells, contributing to ethanol-induced intestinal barrier dysfunction Am J Physiol Gastrointest Liver Physiol 2014 Apr 15;306(8):G677-85.
Therefore, one would expect loss of function studies to determine which MSC is responsible for the calcium increases observed and some insight into the molecular mechanism mediating junctional RhoA activation.
- Figure 4 and 5: Mechanosensitive calcium channel-dependent calcium influx is required for Rho-mediated reinforcement of ZO-1, epithelial barrier function and local junction contraction: GsMTx4 to inhibit MSC-mediated calcium influx results in a decrease in intensity of Rho flares and decreased ZO-1 reinforcement. GsMTx4 makes the leak seen by increases in FluoZin-3 fluorescence more evident after 500s; therefore, active Rho and ZO-1 are analysed up to 504s in Fig 5. However, in Fig S2C GsMTx4 does not change RhoA activation and ZO-1. The scale of time used in the different figures is not clear. Why in Fig 5 data is shown at 1500s but at 400s in Fig. 1-4? Then Fig S1 shows data related to Fig.1 at 1500s. A model is shown in 5H; however, more players involved need to be analysed. Which is the GEF or GAP involved and how does anillin fit in, which was previously shown to activate Rho flares. To better characterise Rho flare regulations will help understanding their physiological relevance.

1st Revision - Authors' Response to Reviewers: December 3, 2021

We thank the reviewers for their helpful comments on our manuscript. We have carefully considered their constructive critiques and have carried out a substantial number of additional experiments and new data analysis. We have added these as well as significant text revisions to our manuscript, resulting in a much stronger revised manuscript. Due to the sizable amount of new data, and in consultation with the editors, we have now formatted the manuscript as a JCB Article. We have addressed each of the reviewers' comments below and made relevant changes to the revised manuscript. Our responses are in blue.

Reviewer #1:

The Miller lab recently (2019) reported that flashes of Rho signaling at cell-cell junctions are responsible for repairing defects in tight junction (TJ) integrity. The present manuscript develops from this finding, to probe the upstream mechanisms that are responsible for activating Rho when TJ defects appear. Prompted by evidence that intracellular calcium signaling can support Rho in other contexts, the authors identify a role for dynamic calcium transients at sites of TJ disruption and repair and implicate mechanosensitive cation channels in this calcium flux, by applying the spider venom toxin GsMTx4. They propose a pathway where mechanosensitive flux of calcium activates Rho to drive TJ repair.

This is an interesting report that is based on data of superb quality. However, I am not sure that it is quite at the level of a JCB Report, at least in its present form.

We thank the reviewer for their positive comment on the quality of the data presented here.

I have two major issues (which are the important ones for me, although there are some specific questions/issues below).

1) Establishing the **causal relationship between calcium and Rho**. The authors reasonably show a role for calcium in Rho signaling, but I am not sure that their linear model (Fig 5H) is fully supported.

I am puzzled that GsMTx4 increased the frequency of Rho flares. In the linear pathway that the authors present (Fig 5H), if calcium signaling were necessary for RhoA to be activated, I would have predicted a decrease in frequency of Rho flares (as well as the decrease in amplitude and duration that the authors document). This disjunction would suggest, to me, that **calcium may not be upstream of Rho activation**, but rather act at some stage **to sustain or amplify the Rho signal**, once it is initiated.

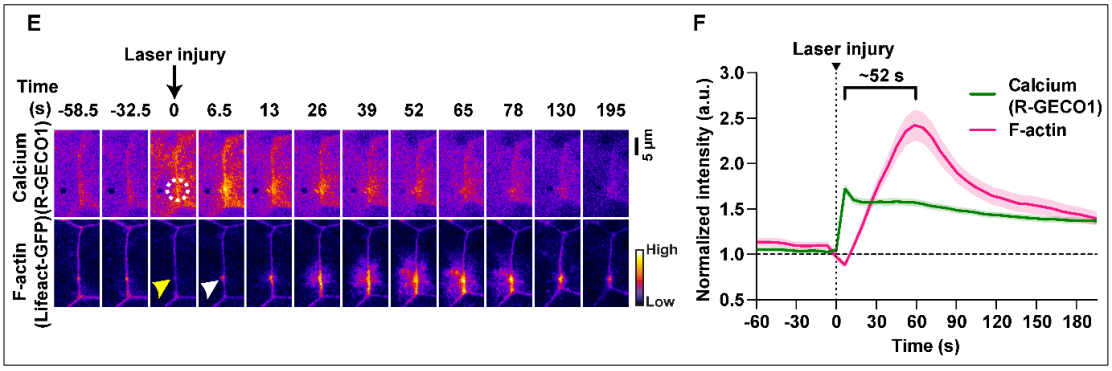
We realize that we were not entirely clear in describing the relationship between calcium and Rho flares at the site of TJ repair. In order to further investigate this issue, we have performed new experiments to test the causal relationship between calcium and Rho, and included the following new data in the manuscript:

1. **Laser injury of tight junctions shows that calcium flash precedes F-actin accumulation:**

   We tested if we could recapitulate the calcium flashes by experimentally inducing a barrier leak, and have now included this data in **Fig. 2 E and F**:

   "To test this, we used 405 nm light to locally injure the TJ in cells expressing probes for F-actin (Lifeact-GFP) and cytosolic calcium (R-GECO1). We have previously shown that laser injury of the TJ causes a leak in the barrier that is repaired similarly to naturally occurring Rho flares (Stephenson et al., 2019). A fluorescent probe that strongly localizes to the junction is necessary to induce a laser injury. Because, F-actin and active RhoA increase nearly simultaneously following TJ leaks (Stephenson et al., 2019), we used Lifeact-GFP to facilitate efficient injury as well as monitor the Rho-mediated repair dynamics.

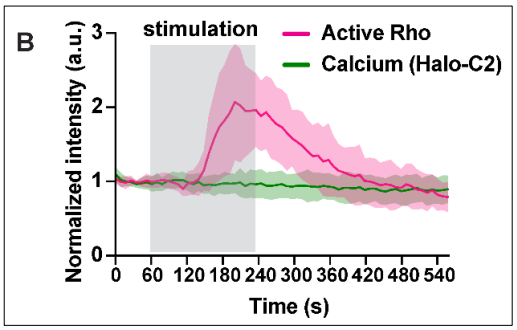
   Upon laser injury, calcium increased locally at the site of the injury followed by an F-actin increase at the site of the injury (Fig. 2, E and F; and Video 5), and the peak of calcium preceded the peak of F-actin by ~52s (Fig. 2 F), comparable to naturally-occurring flares (Fig. 2 D). Moreover, when cells exhibited a short calcium spike that was not sustained, F-actin did not accumulate, resulting in failure to repair the F-actin break induced by laser injury (Fig. 2 G'). Together, these data show that laser-induced TJ leaks result in a local, sustained calcium flash that precedes F-actin accumulation, similar to naturally-occurring barrier leaks."



2. **Optogenetic activation of RhoA at tight junctions shows that local activation of Rho is not sufficient to induce a calcium flash:**

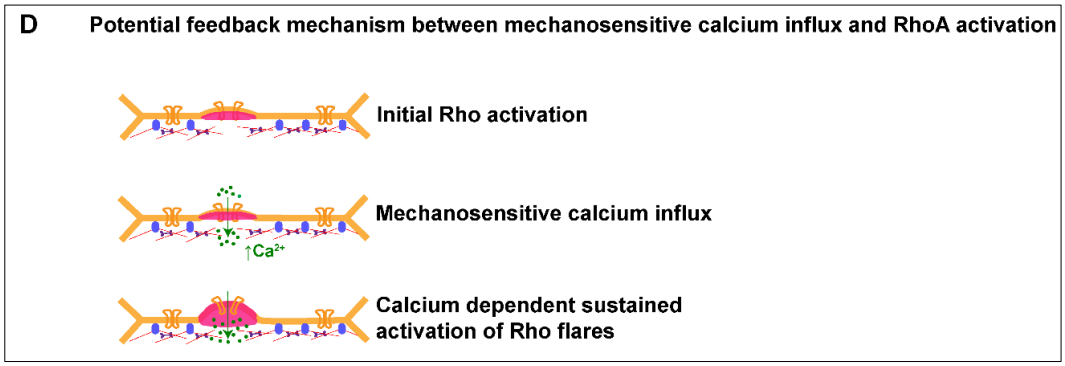
   We tested if optogenetic activation of RhoA at apical junctions can induce a local calcium flash, and have now included this data in **Fig. 3 B, C and C'**:

   "*We next used this system to test if optogenetic activation of RhoA at apical junctions can induce a local calcium flash. RhoA was optogenetically activated in cells expressing a probe for active RhoA (mCherry-2xrGBD) or a probe for membrane calcium (Halo-tagged C2). Light stimulation of a junction from vertex-vertex resulted in RhoA activation at the junction within ~60 seconds after the start of stimulation (Fig. 3, B and C; and Video 7). However, intracellular calcium did not increase upon stimulation (Fig. 3, B and C'; and Video 7). Laser injury of the same junctions resulted in local calcium increase (Fig. 3, D), confirming that the optogenetically activated cells do indeed express a functional calcium probe. Together, this data demonstrates that junctional activation of RhoA is not sufficient to induce a local calcium increase.*"



   Please note that we include also include supplemental data demonstrating that we have effectively adapted the TULIP optogenetic system, which uses 405 nm laser light stimulation to activate RhoA on demand by recruitment of a photo-recruitable GEF to the plasma membrane, for use in *Xenopus* embryos (**Fig. S2**).

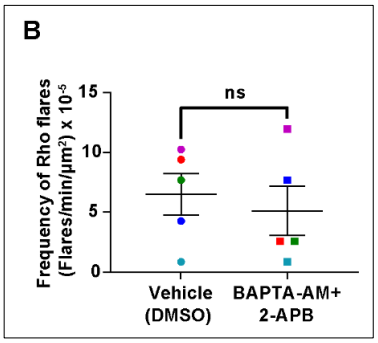
3. **Text changes:**
   Based on our data, while we can show that the *peak* of the calcium increase precedes the peak of Rho flares during naturally-occurring TJ remodeling, and likewise calcium precedes F-actin accumulation during laser-induced TJ leaks, we are unable to resolve the difference in time of *initiation*. So, to reflect the uncertainty in whether this represents a linear model, we have modified the text in the manuscript, edited our model figure **(Fig. 7H),** and added a new figure **(Fig. S5 D, see below)** to address a potential feedback mechanism between RhoA and calcium.



4. **Changed the title of the manuscript:**
We have now changed the title of the manuscript to reflect that calcium flashes are necessary for sustained Rho activation: **"Mechanosensitive calcium flashes promote sustained RhoA activation during tight junction remodeling"**.

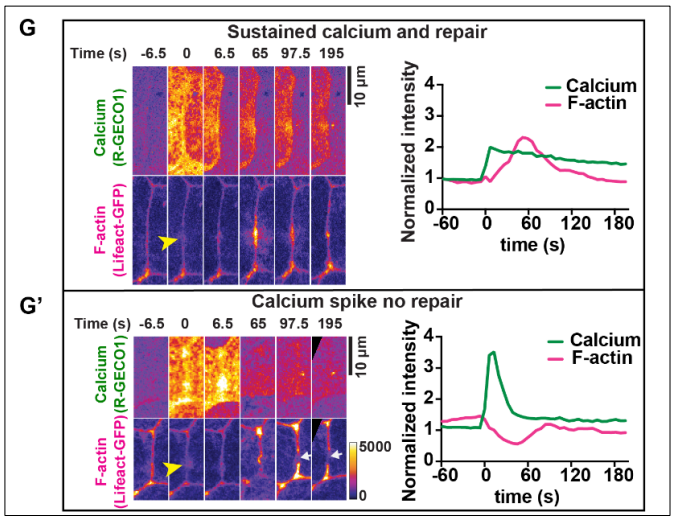
(Along these lines, what does the combination of BAPTA-AM and 2-APB do to the frequency of Rho flares?)

We have now included data in **Fig. S3, B (see below)** showing that BAPTA-AM+2-APB treatment did not significantly affect the frequency of Rho flares, compared to vehicle control (DMSO).



2) New knowledge. I think that it is important to understand how the Rho flares are evoked, but the present analysis applies currently-available concepts (from wound healing, cell migration etc) to the specific instance of TJ repair. Given the prevalence of this model, it is perhaps not surprising that it works in the present instance.

We respectfully disagree with the reviewer that no new knowledge is presented here for the following reasons: 1) During wound healing, the plasma membrane is damaged, leading to a huge inrush of extracellular calcium (no evidence of MSC involvement), so this is quite a different mechanistic framework. 2) For cell migration, the connections between calcium signaling and Rho activation remain mechanistically unclear - except for some understanding in metastatic cancer cells (Pardo-Pastor et al., 2018). Specifically, we are not aware of literature that shows spatiotemporal signaling involving a local calcium increase and Rho activation during cell migration - except for in individual neurons that elicit a local calcium influx at focal adhesions during cell migration (Ellefsen K et al., *Commun Biol.*, 2019). 3) As we discuss in the manuscript, the dynamics and spatial origin of different types of intracellular calcium transients lead to distinct downstream signaling pathways (calcium flashes vs. calcium waves vs. calcium spikes). To highlight the importance of the duration of calcium transients on downstream signaling, we now show that calcium flashes that are not sustained (calcium spikes) fail to illicit proper F-actin accumulation and junction repair, as shown in **Fig. S1 G and G' (see below).** Thus, our findings showing that local calcium flashes are required for sustained activation of RhoA and TJ repair by promoting actomyosin-mediated contraction at the junctions is indeed new knowledge.



For a JCB Report, I would ask for some new conceptual insights. One line to pursue could be the issue of calcium and RhoA activation that I raised above. Another would be to probe the notion that **changes in membrane tension are key at sites of TJ dysfunction**. The authors base this hypothesis on observed deformations of the apical membrane at sites of dysfunction and the loss of a potential actin-membrane coupler in ZO proteins. But this is not addressed in greater depth. (One could imagine, for example, that loss of membrane-actin coupling would decrease the tension that is applied to the bilayer from the cortex - and the protrusions reflect a relaxed ballooning of the membrane.) FRET sensors for membrane/bilayer are now available and could be tried (though I appreciate that their application is not a trivial matter).

This is an excellent point, and we agree with the reviewer that testing the membrane tension at the sites of TJ dysfunction and repair will be a key addition to the mechanism. We pursued two strategies to address this question:

1. We investigated the feasibility of using a membrane tension probe such as Flipper-TR, which is imaged by fluorescence lifetime imaging (FLIM). After consulting with experts at our microscopy core, we concluded this experiment is not feasible in *Xenopus* embryos given the dynamic nature of the embryo (a stable sample is needed to correctly interpret FLIM), and the rapid timescale on which Rho flares occur.

2. In order to test whether loss of ZO-1 prior to Rho flares indicates membrane-actin detachment, we utilized a probe that detects the enrichment of membrane proximal actin, called MPact (membrane proximal actin) (Bisara et al., *Science*, 2020). The probe consists of a fluorophore tagged with F-tractin (F-actin probe) on the N-terminus and a CaaX motif (which gets farnesylated and inserted into the plasma membrane) on the C-terminus. In the absence of membrane-proximal actin, the probe will diffuse in the plane of the membrane like a standard membrane probe. In areas of dense membrane-proximal actin, the probe's diffusion is limited by F-tractin's interaction with F-actin, and MPact signal is locally increased (see **panel A** below).

   We co-expressed BFP-farnesyl and F-tractin-mNeon alongside MPact-mCherry. BFP-farnesyl and F-tractin-mNeon were mostly uniform along cell-cell junctions. We predicted that MPact would localize similarly, with loss of signal occurring before Rho flares, like what is seen for ZO-1. However, we observed that on some junctions, MPact localizes strongly along the length of the junction with intermittent gaps (white arrows, **panel B** below). On other junctions, MPact is relatively low overall with intermittent bright puncta (yellow arrows, **panel B** below).

   Interestingly, we have observed that Rho flares tend to occur between bright puncta of MPact (**panel C** below). Furthermore, MPact puncta move towards each other as the junction contracts locally, and sometimes MPact intensity also increases between the puncta during the flare, suggesting a

reinforcement of membrane proximal actin (**panel D** below). However, unlike ZO-1 breaks, gaps in MPact signal are not good predictors of where Rho flares will occur, and remodeling of MPact prior to flares is not obvious to us at this stage.

While these results are interesting, we believe they deserve more thorough examination and rigorous quantification than we can complete for this manuscript. In their current state, these results don't conclusively support or oppose the hypothesis that membrane tension is altered during or leading up to Rho flares, and thus we do not feel comfortable including these data in the manuscript.

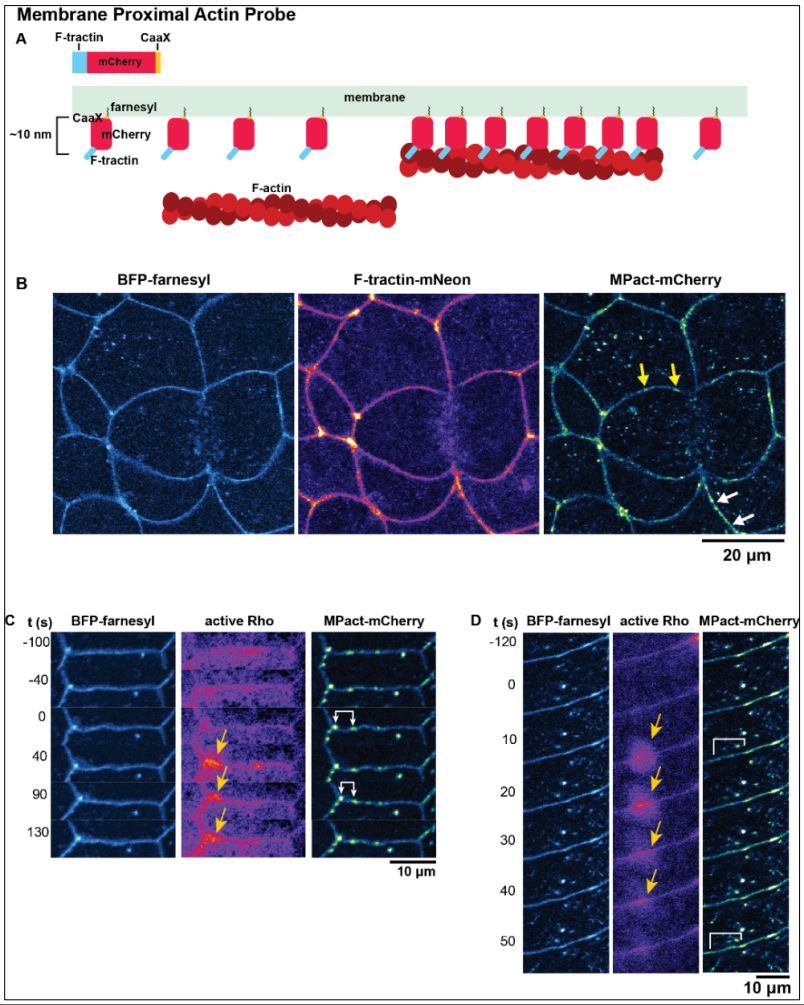
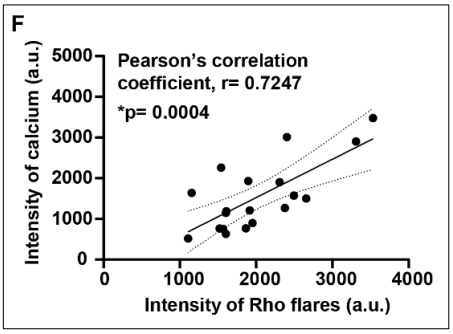


Figure legend: A. Schematic of MPact probe. mCherry is flanked by F-tractin on the N-terminus and a CaaX motif on the C-terminus. Once translated, the CaaX motif is farnesylated and the probe is inserted into the membrane. Probe diffusion is reduced in the presence of membrane-proximal actin (within ~10 nm of the membrane), resulting in locally increased fluorescence intensity. **B**. Co-expression of BFP-farnesyl, mNeon-F-tractin, and MPact-mCherry. Whereas BFP-farnesyl and F-tractin-mNeon are relatively uniformly distributed along cell-cell junctions, MPact intensity is high on some junction with occasional breaks (white arrows) and low on other junctions with occasional bright puncta (yellow arrows). **C.** A Rho flare occurs between two bright puncta of MPact. As the flare occurs and the junction contracts, the puncta move towards one another without significant change in the junctional MPact signal (compare brackets at time 0 s and 90 s). **D.** A Rho flare occurs at a site of low MPact intensity, and MPact increases uniformly over the course of the flare (compare brackets at time 10 s and 50 s).

Specific (more technical) points

a) Fig 2E. Can the authors analyse the relationship between calcium flash and Rho response statistically? At least by eye, I do see some high-ish calcium responses that don't give very impressive RhoA responses and lowish calcium responses that seem to link to large RhoA responses.

We agree with the reviewer that our plot showing the relationship between the area under the curve for calcium and Rho flares was not very clear. We have now improved our analysis, by statistically analyzing the relationship between the intensity of calcium and intensity of active Rho during individual Rho flares using correlation analysis and reporting the Pearson's correlation coefficient as shown in **Fig. S1F** (see below) and in the manuscript text.



b) Why did the authors inject GsMTx4? Can it be added to dechorionated embryos to shorten the incubation time? (To reduce secondary effects that might have arisen from relatively prolonged MSC inhibition.)

We injected GsMTx4 due to: 1) the technically challenging aspect of vitelline removal and the fact that the embryos become very delicate after the vitelline is removed and 2) evidence from the literature showing that acute addition of GsMTx4 to developing *Drosophila* embryos induces a strong calcium influx as a nonspecific effect (Hunter et al., *Development,* 2014). Additionally, other published studies use overnight GsMTx4 treatment (e.g. MDCK cells were treated with GsMTx4 overnight to block MSCs in Takeuchi et al., Current Biology, 2020). Therefore, based on this evidence and especially to avoid the nonspecific effect of acute addition of GsMTx4, we injected GsMTx4. We show that this longer incubation with GsMTx4 did not significantly alter the intensity of junctional active Rho, junctional actomyosin, or E-Cadherin **(Fig. S4)**.

Reviewer #2:

Varadarajan et al study the role of intracellular calcium on epithelial barrier maintenance during development. This work is a continuation of previous findings from the Miller lab on epithelial barrier repair in actively remodelled epithelia (Stephenson et al 2019). Using Xenopus embryos, high resolution live imaging and pharmacological treatments, the authors show that intracellular calcium increase follows paracellular leaks. Furthermore, the authors show that intracellular calcium increase is dependent on the activity of mechanosensitive calcium channels and is necessary for Rho-flare mediated TJ repair. Lastly, the authors show that the mechanosensitive channel mediated calcium influx is necessary for epithelial barrier maintenance during normal development.

The strength of this work comes from the employment of high-resolution live imaging which led to the identification of a novel function of intracellular calcium. The identification of a novel role for intracellular calcium in epithelial barrier maintenance should be of interest to researchers across a broad range of disciplines.

We thank the reviewer for recognizing the strength of the live-imaging, the novelty of our work, and the interest to a broad range of researchers.

Despite the novelty of this work, there are several issues that need to be addressed.

Major points:

1) One of the claims of this work is that intracellular calcium increase precedes RhoA activation during tight junction remodeling and is required for RhoA activation. However, the data presented in the manuscript are not consistent with this notion. Specifically, while the authors argue that calcium flashes precede RhoA activation at sites of tight junction breakage, in Figure 3B, 4C and Movie 2(600sec) there is a clear enrichment of active RhoA before the appearance of calcium elevation. I do not know if this has to do with the sensitivity of detection or kinetics of the probe however even when using the faster calcium reporter (Fig 2D and F) there is an initial increase in RhoA which is synchronous with the calcium elevation. Overall, I am not convinced that calcium elevation does in fact precede RhoA activation and it's just as likely that the opposite is true.

Please see the response to Reviewer #1 above. Briefly, we have performed two additional lines of experiments to address the timing and causal relationship between calcium and Rho. 1) Laser injury of tight junctions shows that the peak of the calcium flash precedes the peak of F-actin accumulation. 2) Optogenetic activation of RhoA at tight junctions shows that local activation of Rho is not sufficient to induce a calcium flash. While our quantification clearly shows differences in the peak levels of calcium and Rho/F-actin, as the reviewer points out, it is harder to resolve the difference in the initiation of elevation. The local optogenetic activation of Rho data indicates that something more than local Rho activation alone is required to initiate a calcium flash (our data suggests that is junction elongation and MSC activation). To reflect the uncertainty of whether the calcium elevation does in fact precede the initiation of Rho activation, we have backed off on language claiming that the intracellular calcium increase precedes Rho activation throughout – in our title, in the text, and in our model figure.

Additionally, in Figures 3C, 4D and 5E there is enrichment of active RhoA at the site of tight junction breakage (albeit lower in intensity) in the absence of calcium elevation. This shows that the RhoA activation at these sites isn't calcium-dependent but may be enhanced by calcium elevation.

We agree with the reviewers that our results point to a mechanism where calcium promotes sustained Rho flare activation during tight junction remodeling. Initial activation of Rho at the junctions that is short-lived and low in intensity is not sufficient to induce junction contraction. Sustained activation of Rho at the junctions is dependent on calcium influx via MSCs for efficient contraction of junctions and reinforcement of ZO-1. We have now modified our text in the manuscript and edited our model figure to reflect this thinking.

The authors should examine the possibility that initial RhoA activation precedes calcium influx and that subsequent calcium influx, possibly triggered by RhoA, further stimulates RhoA. Is RhoA activation necessary for calcium flashes during tight junction remodelling?

The authors could use an optogenetic construct to activate RhoA specifically at the tight junctions to see if that elicits calcium and use RhoA/Rock inhibitors to see if they affect calcium flashes.

We thank the reviewer for this valuable suggestion. To pursue this idea, we have modified the TULIP optogenetic system (Strickland et al., *Nature Methods*, 2012) for use in *Xenopus* to activate RhoA with high spatiotemporal specificity (**Fig. S2**). We used this system to test if optogenetic Rho activation is sufficient to cause a calcium flash, and found that activation of RhoA at apical junctions did not result in a calcium increase **(Fig. 3, and Video 7).** This data shows that local RhoA activation by itself is not sufficient to mediate calcium influx.

With regard to using Rho and ROCK inhibitors: 1) We have previously tried inhibiting RhoA using a cell permeable Rho inhibitor (C3 transferase); however, this leads to global disruption of cell-cell junctions and thus doesn't allow us to address questions related to Rho flares, 2) ROCK inhibitors (Y-2763 and H1152) inhibited junction contraction following Rho flares, but had no effect on the activation of Rho flares (Stephenson et al., 2019). As we show here, junction contraction initiates following the peak of the calcium flash, so we are not convinced this experiment will help us test the effect of Rho flares on calcium flashes.

Finally, can RhoA activation rescue the defective tight junction repair in embryos treated with GMSTX4 of 2AP+BAPTA? Again, an optogenetic construct to activate RhoA specifically at the tight junctions could be used here.

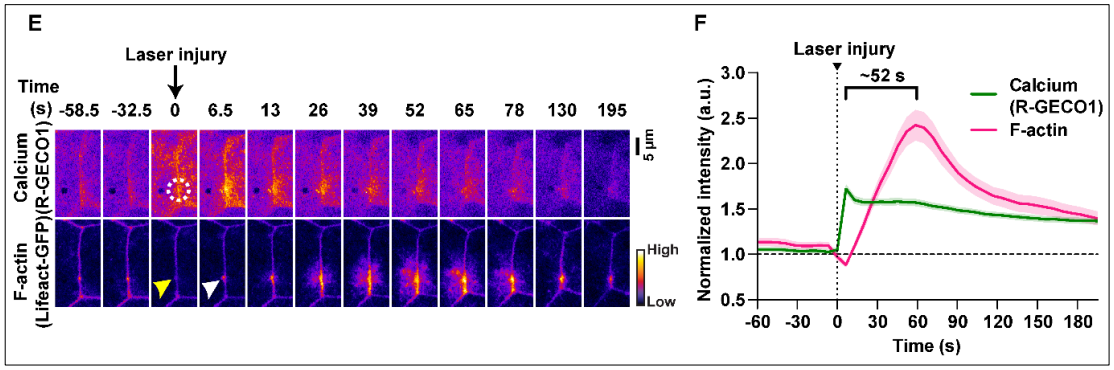
This would be a cool experiment, but is technically very challenging to achieve for the following reasons: 1) while optogenetics is a versatile system to activate RhoA on demand, a current limitation of the system is that the blue and green channels are used for the optogenetic system, thereby limiting the open channels that can be used for other tagged proteins/probes. Therefore, with our current imaging tools, we are unable to co-image barrier leaks (FluoZin-3 is imaged in the green channel) and active RhoA in embryos expressing optogenetic constructs. 2) Tight junction breaks are local and hard to detect while live imaging, thereby making it very challenging to find the region of interest and to optogenetically activate RhoA at the right time consistently between experiments.

2) The authors show a clear correlation between tight junction leakages and calcium flashes. However, they don't directly assess the impact of tight junction leakage on intracellular calcium. The authors could examine the frequency of calcium flashes in control embryos and embryos treated with Latrunculin A which leads to increase of tight junction leakage events (Stephenson 2019). Ideally to directly establish a cause and effect relationship, calcium levels can be examined after TJ laser ablation.

Again, we thank the reviewer for this excellent suggestion. When embryos are treated with Latrunculin A, this leads to major leaks in the tight junction due to depolymerization of junctional actin (Stephenson et al., 2019). Major disruption of cell-cell junctions with Latrunculin A would lead to multiple calcium flashes and calcium waves, making it hard to interpret the impact of tight junction leakage on intracellular calcium. Further, it is likely that weakening of the cortical actin will affect the function of mechanosensitive calcium channels through modulation of membrane tension. For those reasons, we decided to focus on TJ laser ablation instead of Latrunculin A treatment and have now included this data in **Fig. 2 E and F**:

"We tested if we could recapitulate the calcium flashes by experimentally inducing a barrier leak. To test this, we used 405 nm light to locally injure the TJ in cells expressing probes for F-actin (Lifeact-GFP) and cytosolic calcium (R-GECO1). We have previously shown that laser injury of the TJ causes a leak in the barrier that is repaired similarly to naturally occurring Rho flares (Stephenson et al., 2019). A fluorescent probe that strongly localizes to the junction is necessary to induce a laser injury. Because, F-actin and active RhoA increase nearly simultaneously following TJ leaks (Stephenson et al., 2019), we used Lifeact-GFP to facilitate efficient injury as well as monitor the Rho-mediated repair dynamics.

Upon laser injury, calcium increased locally at the site of the injury followed by an F-actin increase at the site of the injury (Fig. 2, E and F; and Video 5), and the peak of calcium preceded the peak of F-actin by ~52s (Fig. 2 F), comparable to naturally-occurring flares (Fig. 2 D). Moreover, when cells exhibited a short calcium spike that was not sustained, F-actin did not accumulate, resulting in failure to repair the F-actin break induced by laser injury (Fig. 2 G'). Together, these data show that laser-induced TJ leaks result in a local, sustained calcium flash that precedes F-actin accumulation, similar to naturally-occurring barrier leaks."

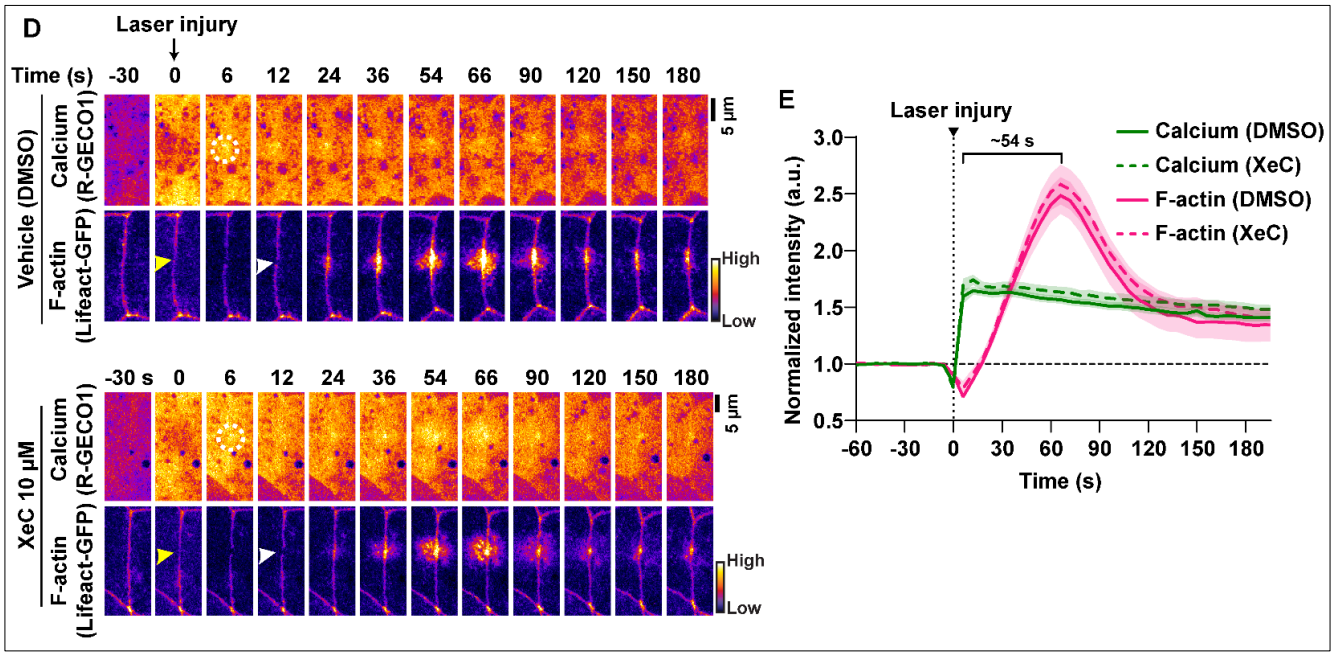


3) The calcium regulated protein PKC has been shown to regulate tight junction assembly and the authors show that the C2 domain of PKC localises at the sites of tight junction leakages. Therefore, PKC might be a downstream effector of calcium during epithelial barrier maintenance. It would be interesting to pharmacologically inhibit PKC to examine the role of PKC in the context of breakage repair.

We agree that this is an interesting possibility. We intend to explore the role of PKC as a potential downstream effector of calcium in tight junction repair in the future, but in consultation with the editors, have decided this direction is outside the scope of our current work.

4) The data presented by the authors show that mechanosensitive calcium channels are responsible for localised calcium influx during paracellular leakages (localised C2-GFP recruitment) and are necessary for tight junction repair and epithelial barrier maintenance. Calcium flashes appear throughout the cell when imaged with the GCAMP6 calcium sensor. This uniform elevation of calcium within cells suggests that IP3 mediated calcium influx from the ER contributes to calcium flashes. This is supported by the fact that 2APB an IP3R inhibitor prevents calcium flashes during tight junction leakage. The authors should discuss or if possible test the possibility that an initial calcium entry through MSCs activates PLC near the plasma membrane, leading to the production of IP3s and subsequent activation of IP3R. Pharmacological PLC inhibition followed by examination of calcium flashes during tight junction remodelling will be beneficial. This will be a great addition to the manuscript since it will reveal if there is a need for production of IP3 downstream of MSCs for the generation of calcium flashes.

We thank the reviewer for this valuable point, and we agree that it would be an important addition to the manuscript. While 2-APB blocks IP_3R mediated calcium release, it has also been reported to block mechanosensitive TRP channel-mediated calcium influx (Takeuchi et al., 2020; Zhang et al., 2017). Therefore, we utilized a more specific IP_3R blocker, Xestospongin C, to test whether IP_3-mediated calcium release from the ER contributes to calcium flashes. We now show that blocking IP_3R had no significant effect on the calcium flash, nor the downstream F-actin-mediated junction contraction when compared to vehicle (DMSO) controls **(Fig. S3, D and E, see below)**. Further, we observed a similar effect on naturally-occurring calcium flash and Rho flares (data not shown).



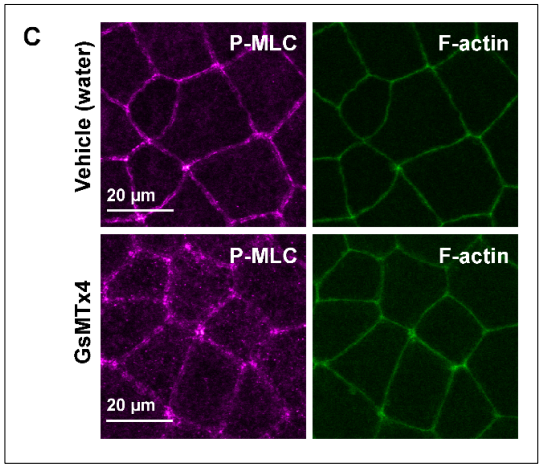
Please note that we also included control data demonstrating that Xestospongin C effectively blocks IP_3-mediated calcium release from the ER in the context of wound healing in *Xenopus* (**Fig. S3 C-C”).**

5) In Figure 2E the authors present a correlation between the levels of calcium and RhoA activation. Plotting calcium levels against active RhoA levels and analysis with a Pearson's correlation coefficient test is the proper way to quantitatively assess if there is a correlation between calcium levels and RhoA activity.

See response to Reviewer #1 on the same point.

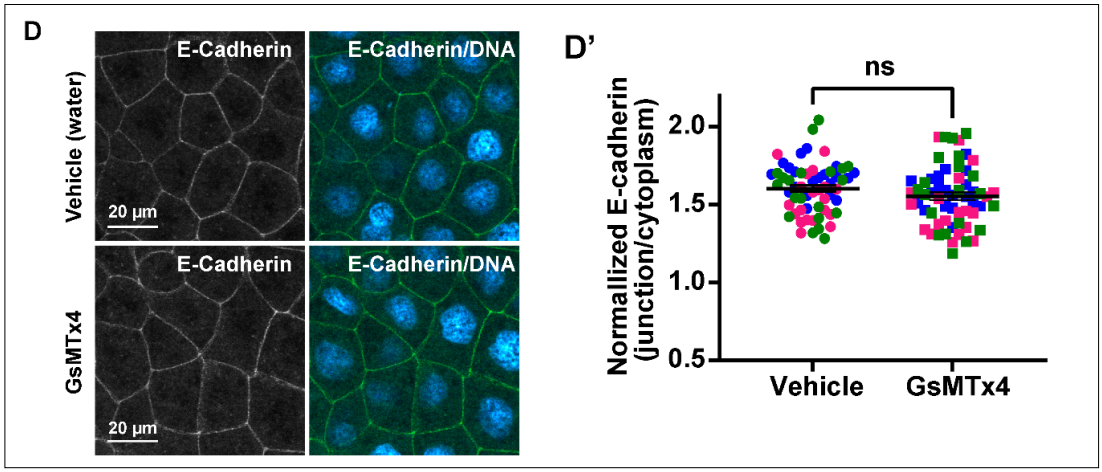
6) The authors argue that GMSTX4 treatment does not affect cell junctions. However, in contrast to the quantification in Fig. S3D', in Figure S3D PMLC appears dramatically reduced in GMSTX4 treated embryos. The reduction of PMLC in GMSTX4 treated embryos shown in Figure S3D argues that cell-cell junctions are affected by GSMTX4 treatment.

We apologize for not including an image representative of the quantitative data of n=45 junctions from 3 independent experiments as shown in **Fig. S4 C'.** We replaced that image with a more representative image as now shown in **Fig. S4 C (see below).** Additionally, a different co-author repeated independent quantification of the intensity of P-MLC and F-actin in control and GsMTx4-treated embryos, confirming that the results agree, and neither P-MLC nor F-actin junctional intensity are affected by GsMTx4 treatment. We do notice a slight redistribution of P-MLC around junctions in GsMTx4-treated embryos, and this is reflected in the statistically significant different in the ratio of P-MLC (junction/cytoplasm).



The authors should stain control and GMSTX4 treated embryos for cell-cell junction markers C-cadherin, b-catenin and or a-catenin to make sure that GSMTX4 treatment does not affect cell-cell junctions.

We have now included data in **Fig. S4, D and D' (see below)** showing that GsMTx4 treatment did not affect E-Cadherin, a marker for adherens junction, compared to vehicle control (water).



7) The authors claim that defective epithelial barrier function (Figure 5A) results from the accumulation of defective repair of local leaks in GMSTX4 treated embryos. Another possibility may be that in GMSTX4 treated embryos the frequency of tight junction leaks increases, especially if cell-cell junctions are affected as shown in PMLC levels in Fig. S3D. To explore this possibility the authors could quantify the frequency of leakage events in control and GMSTX treated embryos. If the frequency of leakage events is unaffected then global epithelial barrier defects stem from the accumulation of defective repair of local leaks. If the frequency of leakage events is increase in GMSTX4 treated embryos, then the global tissue barrier defect stems from both increase of leakage events and accumulation of defective local repairs.

Though we agree with the reviewers that it would be ideal to quantify the number of local leaks, the current technical challenge of measuring the frequency of local barrier leaks over increasing global barrier leak limits our ability to reliably quantify the local barrier leaks in GsMTx4-treated embryos. So, we used Rho flares as an indication of sites of local leaks to get a reliable quantification reporting the frequency of local leaks in control and GsMTx4 treated embryos. We have now included this explanation in the manuscript as follows:

"*As local barrier leaks were followed by Rho flares in control embryos, we used Rho flares as an indicator of sites of local leaks (Stephenson et al., 2019), and analyzed the frequency of Rho flares in vehicle- and GsMTx4-treated embryos.*"

8) In Figure 3a the authors use 2APB+BAPTA AM to block calcium flashes. What happens to calcium flashes when the embryos are treated with only 2APB?

As described above, it has been reported that 2-APB blocks mechanosensitive TRP channel-mediated calcium influx in addition to IP_3R-mediated calcium release (Takeuchi et al., 2020; Zhang et al., 2017). Therefore, we blocked ER-mediated calcium release using a specific IP_3R blocker, Xestospongin C. We now show that blocking IP_3R had no significant effect on calcium flashes or the downstream F-actin-mediated junction contraction compared to vehicle control (DMSO).

Specific concerns:

1) Maybe the authors can swap Fig1 C and D to better follow the flow of the text?

This is a good suggestion, and we have switched the order of the figures as suggested (Fig. 1, C and D).

2) In Figures 1B and 2d the authors use a different approach to quantify Calcium (C2) and calcium (GCAMP6). Is there a reason for this? why is calcium signal quantification different?

The GCaMP6m and C2 calcium probes detect the level of intracellular calcium based on different mechanisms. GCaMP6m is a cytoplasmic soluble calcium probe where the fluorescence increases when bound to calcium ions, and the probe has a high signal-to-noise ratio. However, C2 is recruited to the plasma membrane upon binding calcium ions, and the probe has high background noise compared to GCaMP6m - even in the absence of a calcium increase. To account for the high background with the C2 probe, we subtracted the background signal from the intensity at the flare.

3) In some examples like Fig2a, 2c-d only one cell next to the junction displays a calcium flash. Can the authors discuss this? Is calcium only required in one of the two cells repairing the TJ? Is there a difference in repair kinetics if only one of the two cells displays calcium elevation? Could the authors introduce the RhoA sensor in a mosaic pattern and examine RhoA activation at breakpoints in cells that do display calcium vs ones that do not?

We thank the Reviewer for pointing to the asymmetric calcium increase. We find this interesting as well, and have previously shown that there is an asymmetric increase in active Rho, F-actin, and Myosin II during tight junction reinforcement (Stephenson et al., 2019). Further, we have detected ZO-1 loss in both cells using mosaic expression of fluorescently-tagged ZO-1 (unpublished). We hypothesize that the asymmetric calcium increase could be associated with the degree of ZO-1 loss in one cell versus the other. While examining the asymmetric calcium increase in embryos expressing mosaic Rho probes is a great idea, this type of experiment is technically challenging and time consuming. Given that Rho flares are sporadic, it can be difficult to capture Rho flares on

mosaically-labeled junctions, and capturing cells with an asymmetric calcium increase as well adds an additional layer of difficulty. Therefore, we were unable to test this idea during the timeframe for this revision, but it remains an interesting idea for future studies.

4) In figure 4a the authors show that membrane blebs correlate with RhoA flares, suggesting increase in local membrane tension. Is there any direct evidence that membrane blebs are sites of increased tension. Citing previous work like this one https://www.pnas.org/content/106/44/18581 will strengthen the authors' hypothesis.

We now cite literature showing that blebs are indeed sites of increased PM tension, and have discussed a potential mechanism for the initiation and growth of membrane protrusion correlated with Rho flares in the Discussion as follows:

"Rho flares following TJ leaks are associated with apical membrane protrusions (Fig. 6), displaying a change in membrane curvature and thereby a possible change in membrane tension (Stephenson et al., 2019). However, it is not clear if the membrane protrusions observed during Rho flares are bleb-like protrusions devoid of actin or lamellipodia-like actin-based protrusions. Bleb-like protrusions occur where the connection of the PM to the underlying actin cortex is weakened or lost (Charras, 2008), whereas lamellipodia-like protrusions occur when Arp2/3-mediated branched actin pushes the membrane outward (Taha et al., 2014). While the growth phase of typical blebs is devoid of cytoskeletal elements in the protrusion and pushed out due to intracellular pressure, the membrane protrusion observed during Rho flares has a homogenous distribution of active Rho and F-actin (Fig. 6, and S5 B), indicating that Rho flares might follow a combination model, where the initiation phase follows a bleb-like pattern due to the detachment of PM from the cortex on loss of ZO-1, followed by a formin- and Arp2/3-mediated actin-based protrusion model during the growth phase. In agreement with our idea, a recent study shows that initial detachment of PM from the cortex is required for initiation of both bleb-like and F-actin-mediated protrusion (Welf et al., 2020). Given that membrane protrusions are reported to be sites of increased PM membrane tension (Shi et al., 2018), and that the growth phase of membrane protrusion is dependent on the cytoskeletal-mediated cortical tension ***(Tinevez et al., 2009)****, it will be of interest in the future to understand the effect of TJ remodeling on local PM tension and thereby the modulation of MSCs."*

5) In some of the time-lapse movies there are calcium flashes in cells away from tight junction leaks. Can the authors comment on this?

Rho flares mediate junction contraction at the site of TJ leaks, and this often leads to elongation of a nearby junction, followed by a TJ break, calcium flash, and Rho flare. For accurate measurement of calcium flash dynamics using the cytoplasmic soluble calcium probe (GCaMP6m), Rho flares were quantified only in cells that exhibited an isolated Rho flare that was not interrupted by a multicellular travelling calcium wave or a calcium flash from a nearby cell for 500 s prior to and after the initiation of the Rho flare.

6) The authors are compressing the embryos during imaging. Are the breaks in part induced by this slight compression?

The Reviewer raises a good point, and we have also thought about this issue. We predict that the breaks are not caused by the slight compression of the embryos for the following reason: If breaks were induced by compression caused upon mounting of embryos, we would expect that the occurrence of TJ breaks and Rho flares would preferentially happen at the start of the movie following mounting. However, we see that TJ breaks and Rho flares happen sporadically throughout the course of the movie (~30-40 minutes).

Reviewer #3:

Cell-cell junctions respond to different stimuli including mechanical ones that are known to regulate tight junction assembly/integrity via different types of signalling pathways including calcium increases and RhoA-regulated actomyosin remodelling. Previously, the Miller group has found that local leaks in the barrier are rapidly repaired by localized transient activation of RhoA that they call Rho flares.

This paper asks how Rho flares are initiated. They discover that intracellular calcium flashes occur in Xenopus laevis epithelial cells undergoing Rho flare-mediated remodelling of ZO-1. Depletion of intracellular calcium or inhibition of mechanosensitive calcium channels (MSC) reduced the amplitude of calcium flashes and diminished the activation of Rho flares. They propose a model in which MSC-dependent calcium flashes are important for epithelial cells to sense and respond to local barrier leaks induced by mechanical stimuli.

This paper is using a similar methodology as described by the same group in two previous papers to study Rho flares. The present paper remains at a descriptive level and solely relies on drugs/inhibitors to analyse the molecular mechanism of Rho activation. No attempts are made to identify the actual channel relevant for the calcium influx nor the molecular mechanism leading from calcium increases to RhoA activation. Given what is known about the roles of calcium and RhoA in tight junction formation, the current manuscript does not represent a significant advance. For example, ethanol-induced [Ca(2+)]i release, mediated by stimulating IP3R-gated Ca(2+) channel, activates Rho/ROCK in Caco-2 cells, contributing to ethanol-induced intestinal barrier dysfunction Am J Physiol Gastrointest Liver Physiol 2014 Apr 15;306(8):G677-85.

We respectfully disagree with the reviewer that the role of calcium and RhoA in tight junction remodeling is already known. The role of intracellular calcium has been studied during the biogenesis of tight junctions following a calcium switch assay, which is a very different (and arguably less physiologically relevant) scenario. During a calcium switch assay, both adherens junctions and tight junctions are completely disassembled upon depletion of extracellular calcium. Upon re-addition of extracellular calcium, tight junction biogenesis follows the formation of adherens junctions. Moreover, the paper mentioned by the reviewer (Elamin et al., 2014), shows exactly the opposite of our mechanism – in the Elamin paper, Rho activation leads to barrier dysfunction! We instead show that local MSC-mediated calcium influx is associated with local Rho activation and actomyosin-mediated junction contraction to successfully reinforce the TJ proteins without disrupting tight junction function more broadly. Further, Elamin et al. show that ER-mediated calcium increases the phosphorylation of MYPT1, and not the dynamics of RhoA activation.

Therefore, one would expect loss of function studies to determine which MSC is responsible for the calcium increases observed and some insight into the molecular mechanism mediating junctional RhoA activation.

We agree with the reviewer that it would certainly be of interest to identify the MSC responsible for the local calcium increase. Multiple MSCs including Piezo and TRP channels are expressed in epithelial cells. GsMTx4 blocks both Piezo1 and TRPC6 channels, and *Xenopus laevis* gastrula-stage embryos express two alloalleles (.S and .L) of both Piezo1 and TRPC6 (Session et al., *Nature*, 2016). Therefore, examining the role of each of these channels will take significant time and is outside the scope of the current manuscript.

Figure 4 and 5: Mechanosensitive calcium channel-dependent calcium influx is required for Rho-mediated reinforcement of ZO-1, epithelial barrier function and local junction contraction: GsMTx4 to inhibit MSC-mediated calcium influx results in a decrease in intensity of Rho flares and decreased ZO-1 reinforcement. GsMTx4 makes the leak seen by increases in FluoZin-3 fluorescence more evident after 500s; therefore, active Rho and ZO-1 are analysed up to 504s in Fig 5. However, in Fig S2C GsMTx4 does not change RhoA activation and ZO-1. The scale of time used in the different figures is not clear. Why in Fig 5 data is shown at 1500s but at 400s in Fig. 1-4? Then Fig S1 shows data related to Fig.1 at 1500s.

We apologize that we did not clearly explain the difference between local and global changes in the barrier. In Fig. 7, A and B, we are exploring the effects of GsMTx4 on the overall integrity of the tight junction barrier, and not looking specifically at the site of local leaks as in Fig. 1-6, and Fig. 7, D-E. For unbiased quantification of the global integrity of the barrier between control and GsMTx4 treated embryos, we measured the whole field intensity of FZ-3 (the dye that detects barrier leaks) from time 0 s, which is right after the embryos are mounted in zinc-containing media, until 1500 s. As the local leaks happen sporadically throughout the time course of imaging, the timing of local changes is measured before and after a local leak initiates and therefore is on a different timescale than the global measurements in Fig. 7, A and B.

A model is shown in 5H; however, more players involved need to be analysed. Which is the GEF or GAP involved and how does anillin fit in, which was previously shown to activate Rho flares. To better characterise Rho flare regulations will help understanding their physiological relevance.

We agree with the reviewer that it is important to study the role of more players involved in Rho flare regulation including GEFs, GAPs and Anillin. Screening potential GEFs and GAPs that are mediated by calcium is certainly of high interest for future studies, but is outside the scope of this work. We have now speculated about the potential connection between calcium, Rho flares, and Anillin in the Discussion as follows:

"*Alternatively, intracellular calcium increase could sustain RhoA activation by increasing the residence time of RhoA in Anillin dependent manner (Budnar et al., 2019). In agreement with this idea, we previously showed that knockdown of Anillin leads to short-lived Rho flares (Reyes et al., 2014), similar to GsMTx4-treated cells observed in this study. Thus, suggesting that the calcium increases the residence time of junctional active RhoA in Anillin dependent manner by enriching PIP2 at the PM (Wang and Richards, 2012) or binding C2 domain of Anillin (Cho and Stahelin, 2006; Sun et al., 2015).*"

January 6, 2022

RE: JCB Manuscript #202105107R-A

Dr. Ann L Miller
University of Michigan-Ann Arbor
Molecular, Cellular, and Developmental Biology
5264 Biological Sciences Building
1105 North University Ave.
Ann Arbor, MI 48109-1085

Dear Dr. Miller:

Thank you for resubmitting your manuscript with the new title: "Mechanosensitive calcium flashes promote sustained RhoA activation during tight junction remodeling". It was returned to the original three expert reviewers for a full re-review. As you can see from the appended reviews, the reviewers were split in their recommendations, with two supporting publication and one opposed on the basis of insufficient mechanistic insight. Given this difficult editorial decision when faced with divergent reviews from three leaders in the field, our editorial decision is to offer publication after minor revisions.

Two of the original highly expert reviewers felt that their concerns were resolved and recommended acceptance for publication after correction of an incorrect reference to a Fig. 2G', which could not be found. The third leader in the field felt that it did not provide a physiological mechanism, a point that we acknowledge reduces enthusiasm for this study. However, based on the original correspondence appealing the original rejection of this study and the proposed research plan to address the concerns of the three reviewers that we accepted, we do understand the arguments both for and against its novelty. Though we would have dearly liked to have seen additional mechanistic insight, if it is possible to simply resolve the problem of the missing figure panel, we feel that this paper provides enough information for publication in JCB.

To avoid unnecessary delays in the acceptance and publication of your paper, please read the following information carefully.

A. MANUSCRIPT ORGANIZATION AND FORMATTING:

Full guidelines are available on our Instructions for Authors page, https://jcb.rupress.org/submission-guidelines#revised. **Submission of a paper that does not conform to JCB guidelines will delay the acceptance of your manuscript.**

1) Text limits: Character count for Articles and Tools is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, and acknowledgments. Count does not include materials and methods, figure legends, references, tables, or supplemental legends. You are currently just below this limit but please bear it in mind when revising.

2) Figure formatting: Scale bars must be present on all microscopy images, including inset magnifications. Molecular weight or nucleic acid size markers must be included on all gel electrophoresis.

3) Statistical analysis: Error bars on graphic representations of numerical data must be clearly described in the figure legend. The number of independent data points (n) represented in a graph must be indicated in the legend. Statistical methods should be explained in full in the materials and methods. For figures presenting pooled data the statistical measure should be defined in the figure legends. Please also be sure to indicate the statistical tests used in each of your experiments (both in the figure legend itself and in a separate methods section) as well as the parameters of the test (for example, if you ran a t-test, please indicate if it was one- or two-sided, etc.).Also, since you used parametric tests in your study (e.g. t-tests, ANOVA, etc.), you should have first determined whether the data was normally distributed before selecting that test. In the stats section of the methods, please indicate how you tested for normality. If you did not test for normality, you must state something to the effect that "Data distribution was assumed to be normal but this was not formally tested."

4) Materials and methods: Should be comprehensive and not simply reference a previous publication for details on how an experiment was performed. Please provide full descriptions (at least in brief) in the text for readers who may not have access to referenced manuscripts. The text should not refer to methods "...as previously described."

5) Please be sure to provide the sequences for all of your primers/oligos and RNAi constructs in the materials and methods. You must also indicate in the methods the source, species, and catalog numbers (where appropriate) for all of your antibodies.

6) Microscope image acquisition: The following information must be provided about the acquisition and processing of images:
a. Make and model of microscope
b. Type, magnification, and numerical aperture of the objective lenses

c. Temperature
d. imaging medium
e. Fluorochromes
f. Camera make and model
g. Acquisition software
h. Any software used for image processing subsequent to data acquisition. Please include details and types of operations involved (e.g., type of deconvolution, 3D reconstitutions, surface or volume rendering, gamma adjustments, etc.).

7) References: There is no limit to the number of references cited in a manuscript. References should be cited parenthetically in the text by author and year of publication. Abbreviate the names of journals according to PubMed.

8) Supplemental materials: There are strict limits on the allowable amount of supplemental data. Articles/Tools may have up to 5 supplemental figures. At the moment, you are below this limit but please bear it in mind when revising.
Please also note that tables, like figures, should be provided as individual, editable files. A summary of all supplemental material should appear at the end of the Materials and methods section.

9) eTOC summary: A ~40-50 word summary that describes the context and significance of the findings for a general readership should be included on the title page. The statement should be written in the present tense and refer to the work in the third person. It should begin with "First author name(s) et al..." to match our preferred style.

10) Conflict of interest statement: JCB requires inclusion of a statement in the acknowledgements regarding competing financial interests. If no competing financial interests exist, please include the following statement: "The authors declare no competing financial interests." If competing interests are declared, please follow your statement of these competing interests with the following statement: "The authors declare no further competing financial interests."

11) A separate author contribution section is required following the Acknowledgments in all research manuscripts. All authors should be mentioned and designated by their first and middle initials and full surnames. We encourage use of the CRediT nomenclature (https://casrai.org/credit/).

12) ORCID IDs: ORCID IDs are unique identifiers allowing researchers to create a record of their various scholarly contributions in a single place. At resubmission of your final files, please consider providing an ORCID ID for as many contributing authors as possible.

B. FINAL FILES:

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Please revise and format the manuscript and upload materials within 7-14 days. If complications arising from measures taken to prevent the spread of COVID-19 will prevent you from meeting this deadline (e.g. if you cannot retrieve necessary files from your laboratory, etc.), please let us know and we can work with you to determine a suitable revision period.

We thank you again for sending this well-documented study to the Journal of Cell Biology and look forward to hearing about potential resolution of the problem with Fig. 2G'.

With kind regards,

Ken Yamada

Kenneth M. Yamada, MD, PhD
Editor
Journal of Cell Biology

Tim Spencer, PhD
Executive Editor
Journal of Cell Biology

Reviewer #1 (Comments to the Authors (Required)):

I think that the authors have reasonably addressed the issues raised in my earlier review. It is a thorough study, which provides valuable new information, and will undoubtedly be a basis for future work.

Minor points

1. There is a reference to "Fig 2G'" (bottom of p 7 and in quoted in Response to Reviewers), but this is not present in Fig 2.

Reviewer #2 (Comments to the Authors (Required)):

The new data provided by the authors in the revised version of the manuscript and text modification have addressed all my comments.
Therefore, I recommend the publication of this manuscript without further revisions.

Minor comment
Figure 2G' referred in the main text of the revised manuscript does not exist. Probably the authors mislabelled this in the manuscript. From the description, it seems that Fig. 2G' should be replaced by Fig S1G-G' in the main text.

Reviewer #3 (Comments to the Authors (Required)):

Different types of signalling mechanisms, including mechanically stimulating ones, regulate calcium increases and RhoA-mediated actomyosin remodelling, affecting cell-cell junction assembly/integrity, including tight junctions. Previously, the Miller group has found that local leaks in the barrier are rapidly repaired by localized transient activation of RhoA to which they refer as Rho flares.

This manuscript address in more detail how Rho flares are initiated. Intracellular calcium flashes occur in Xenopus laevis epithelial cells undergoing Rho flare-mediated remodelling of ZO-1. Intracellular calcium depletion or inhibition of mechanosensitive calcium channels by drugs reduced the amplitude of calcium flashes and the activation of Rho flares. They propose a model in which mechanosensitive calcium channel-dependent calcium flashes are important for epithelial cells to sense and respond to local barrier leaks.
A similar methodology was described by the same group in two previous papers to study Rho flares. A previous manuscript was rejected because it did not add any significant new advance on the actual mechanosensitive calcium channels that induces Rho flares, only drugs/inhibitors were used to analyse whether calcium precedes Rho activation.

The resubmitted paper does not actually address the main reviewers' concerns on the actual molecular mechanism. The author's reply is very long listing several changes and more experimental details.

1) Changed of the title: "Mechanosensitive calcium flashes promote sustained RhoA activation during tight junction remodelling
2) Laser injury of tight junctions to show that calcium flash precedes F-actin accumulation (Fig. 2 E and F)
3) Optogenetic activation of RhoA at tight junctions to show that local activation of Rho is not sufficient to induce a calcium flash (Fig. 3 B, C and C')
4) Blocking IP3 receptors to show that they are not necessary for the calcium flash, nor the downstream F-actin-mediated junction contraction (Fig. S3, D and E).
5) GsMTx4 treatment did not affect E-cadherin, a marker for adherens junction (Fig. S4, D and D')
6) Several changes for clarifications and an improved model.

Unfortunately, the revision does not add novel mechanistic insight. No loss of function studies to determine the actual relevant channel, whether Piezo1 and/or TRPC6 channels, for the calcium increases and/or which is the actual molecular mechanism after calcium increase to lead to junctional RhoA activation, which is the GEF or GAP involved and/or how does anillin fit in, which was previously shown by the authors to activate Rho flares.
In brief, the revised manuscript does not represent a significant advance in insight into the molecular mechanism nor in the context of a physiologically relevant process. Only a revised version that identified more specifically any of the key players before or after calcium flashes and sustained RhoA activation would provide novel and significant mechanistic insight of interest to a wide cell biology readership.